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# UNIVERSITÀ DEGLI STUDI DI TORINO

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**Root starch accumulation in response to arbuscular mycorrhizal colonization differs among *Lotus japonicus* starch mutants**

Caroline Gutjahr, Mara Novero, Tracey Welham, Trevor Wang, Paola Bonfante

## Abstract

Arbuscular mycorrhizal (AM) fungi are obligate symbionts dependent for completion of their life cycle on plant carbohydrates, which they trade for mineral nutrients. Plant colonization by AM fungi is therefore expected to induce profound changes in plant carbon metabolism. We have previously observed that on one hand starch accumulation increases in responses to pre-symbiotic fungal signals and on the other hand, it decreases in mycorrhizal *Lotus japonicus* roots (Gutjahr et al. in *New Phytol* 183:53–61, 2009). To examine the importance of starch metabolism for AM development, we took advantage of a novel series of *Lotus japonicus* mutants impaired either in starch degradation or in synthesis. Normal AM colonization in all mutants indicated that defects in starch metabolism do not affect AM development and that carbohydrates can be supplied to the AM fungus without a requirement for starch synthesis. Furthermore, our experiments allowed us to characterize root starch dynamics in detail and point to continued turnover of starch in the degradation mutants in the presence of mycorrhiza.

## Keywords

Amyloplast Arbuscular mycorrhizal symbiosis *Gigaspora margarita* *Lotus* Starch Sugar metabolism

## Abbreviations

AM

Arbuscular mycorrhizal

Gwd

Glucan water dikinase

Pgm

Phosphoglucomutase

WT

Wild type

## Introduction

Most vascular land plants form root symbioses with beneficial arbuscular mycorrhizal (AM) fungi that take up inorganic ions (especially phosphate and nitrogen) from the soil and transfer them to their host (Smith and Read 2008). In turn, plants provide these obligately biotrophic fungi with up to 20% of their photosynthetically fixed carbon (Bago et al. 2000). Due to the beneficial effects on plant mineral nutrition, AM symbioses receive increasing attention for their application in sustainable agriculture. Since AM fungi also represent a carbon cost to the plant, it is of utmost importance to characterize and understand the molecular components involved in the mutual exchange of nutrients and their impact on plant physiology and productivity (Sawers et al. 2008). While transcriptomics and genetics studies have provided convincing evidence that the AM fungus plays an important role in plant P and N uptake (Balestrini et al. 2007; Javot et al. 2007; Gomez et al. 2009; Guether et al. 2009a; Branscheid et al. 2010) and indicate how the nutrient status of the plant influences AM development (Javot et al. 2007; Yoneyama et al. 2007; Nagy et al. 2009; Breuillin et al. 2010), information on the molecular components important for carbon transfer to the fungus and carbon metabolism in mycorrhizal plants remains rather patchy (Franken 2010).

Several studies have described metabolic and transcriptional changes in plants in relation to carbon metabolism during AM colonization (Shachar-Hill et al. 1995; Blee and Anderson 2002; Ravnskov et al. 2003; Schubert et al. 2003; Schaarschmidt et al. 2006; García-Rodríguez et al. 2007; Tejeda-Sartorius et al. 2008) and in some cases, the extent to which carbon supply is limiting. For example, transgenic manipulation of invertase levels demonstrated on one hand that hexose supply to roots limits AM colonization (Schaarschmidt et al. 2007a), but on the other hand that AM colonization cannot be increased above a certain threshold by elevating carbon transport to the root (Schaarschmidt et al. 2007b).

Starch is the major storage polymer of carbon in plants (Tetlow et al. 2004). We have recently observed in *Lotus japonicus* that AM colonization affects the root starch economy as the starch content decreases in mycorrhizal as compared to control roots (Gutjahr et al. 2009). Interestingly, prior to colonization in response to AM fungal signals starch accumulates indicating that changes in root carbon metabolism precede mycorrhizal colonization (Gutjahr et al. 2009). However, it remains unclear whether starch accumulation is absolutely required for AM colonization. To investigate the importance of root starch metabolism for AM development we took advantage of the availability of an extensive series of novel *Lotus japonicus* mutants that are either defective in starch synthesis or breakdown (Vriet et al. 2010). Here we show that AM colonization does not depend on root starch accumulation and plant sugars can be supplied to the AM fungus without a detour through starch. We further provide evidence that starch turnover does occur in wild type and some of the degradation mutants, indicating that when starch is present, it is used as a second source of energy for the AM symbiosis.

## Materials and methods

### Plant culture

*Lotus japonicus* (Regel) Larsen ecotype Gifu seeds, wild type (produced at Dipartimento di Biologia Vegetale, Università di Torino, Italy) and mutants SL5035-7, SL4841-4 and *pgm1-4* (SL 4725-4) (produced at John Innes Centre, Norwich Research Park, UK) were surface sterilized and scarified for 3 min in sulphuric acid, washed three times for 15 min with sterile, distilled water and germinated on water agar (0.6%) in Petri dishes. The *Lotus japonicus* mutants, SL5035-7, SL4841-4 and *pgm1-4* have been described previously (Table 1; Vriet et al. 2010).

**Table 1**  
Quantity of mycorrhizal colonization of starch mutants (after Trouvelot et al. 1986)

	Phenotype	F%	M%	a%	A%	<i>P</i> value (F%)	<i>P</i> value (A%)
WT		54.85	13.14	81.41	12.95		
SL5035-7	No starch break down leaves	43.40	11.65	99.20	11.56	0.47	0.92
SL4841-4	No starch break down in leaves	55.00	11.50	84.54	10.56	0.92	0.47
SL4725-4 ( <i>pgm1-4</i> )	No starch synthesis in any part of the plant	89.17	28.80	97.77	28.14	0.36	0.026

F% frequency of mycorrhizal colonization (percentage of segments showing internal colonization), M% intensity of mycorrhizal colonization (average colonization meant as visible coverage of a root segment), a% percentage of arbuscules (average presence of arbuscules within colonized areas), A% percentage of arbuscules in the root system (quantity of arbuscules as referred to the whole root system). *P* values showing significant difference at the 95% level have been obtained by a Kruskal-Wallis test and refer to the comparison with the wild-type, *n* = 5

### Mycorrhizal inoculation

Mycorrhizal colonization of *Lotus japonicus* was induced as described in Novero et al. (2002). Two weeks' old seedlings (pre-germinated on 0.6% water agar) were inoculated with *Gigaspora margarita* Becker and Hall (strain deposited in the Bank of European Glomales as BEG 34) using the Millipore sandwich method (Giovanetti et al. 1993) and grown in a climatic chamber at 21°C during the day, 18°C during the night and with 16 h of light per day. After 4 weeks when full colonization was reached (Novero et al. 2002), plants were harvested at the beginning of the day (2 h of light) and roots cut and stained for observation of mycorrhization or used for starch extraction.

### Fungal staining and quantification of colonization

Five entire root systems of each genotype grown in separate magenta boxes were stained in 0.1% cotton blue (w/v) in lactic acid and mycorrhizal colonization was quantified as described (Novero et al. 2002). For morphological observations, semi-thin root sections were prepared as described (Novero et al. 2002) and stained in 1% toluidine blue.

### Periodic Acid Schiff staining and starch extraction

Amyloplasts were stained by the Periodic Acid Schiff (PAS) reaction as described previously (Gutjahr et al. 2009). For SL5035-7, SL4841-4 and wild type at least five semi-thin microtome sections each from five different plants grown in independent magenta boxes were obtained.

Starch was extracted from five to six samples grown in independent magenta boxes as described (Gutjahr et al. 2009). Each sample was a pool of two root systems which were grown in the same magenta box. The different mutants were grown and analyzed along with wild type at different points in time, revealing a high variability among the independent experiments. The different experiments were therefore analyzed and displayed separately.

## Statistics

Data were analyzed with the statistical software SYSTAT 10. The significance of differences at the 95%-level was assessed using the Kruskal–Wallis test for non-parametric data.

## Results

### Morphology and intensity of mycorrhizal colonization

We hypothesized that defects in starch breakdown and accumulation may interfere with the sugar flow towards the root and impact on the formation of the AM symbiosis. To test this hypothesis, we chose the *Lotus japonicus* mutants SL5035-7 and SL4841-4 that are disturbed in starch breakdown, and SL4725-4 that cannot produce starch in any parts of the plant (Vriet et al. 2010). SL5035-7 and SL4841-4 have been mapped to two different loci on chromosome 1 but the mutated genes are unknown. SL 4725-4 carries a mutation in PHOSPHOGLUCOMUTASE 1 and was named *pgm1-4* (Vriet et al. 2010). All mutants were subjected to colonization by *G. margarita* for 4 weeks, to reach full colonization (Novero et al. 2002). We determined first whether the morphology of the colonizing fungus was changed due to the defective starch metabolism of the mutants. The colonization pattern and the morphology of arbuscules were equivalent to the wild type in all three mutants SL5035-7, SL4841-4 and *pgm1-4* (Fig. 1).

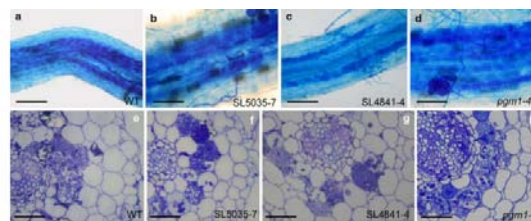


Fig. 1

Patterns of mycorrhizal colonization in root pieces stained with 0.1% cotton blue (upper part) and root cross-sections stained with toluidine blue (lower part) of wild type, SL5035-7, SL4841-4 and *pgm1-4*. Bars: 400  $\mu$ m (a), 200  $\mu$ m (b, d), 300  $\mu$ m (c), 50  $\mu$ m (e, h), 75  $\mu$ m (f), 60  $\mu$ m (g).

Mycorrhizal colonization of the four genotypes was quantified with the estimative method of Trouvelot et al. (1986). Data from 1 cm root segments of five whole root systems for each genotype were taken to calculate the four parameters that display the intensity of colonization by the AM fungus. The frequency of colonization in the wild-type was around 60% as expected for the time point of 4 wpi at which samples were taken (Novero et al. 2002). No significant differences were found among the values assumed by the parameters in the three mutants and the wild type according to the Kruskal–Wallis non-parametric test (Table 1). In conclusion, the mutations SL5035-7, SL4841-4 and *pgm1-4* do not affect AM colonization negatively. However, although not statistically significant at the 95% level, *pgm1-4*, which cannot produce starch, seemed to be slightly more receptive to the AM fungus. Especially the percentage of arbuscules in the root system was more than double as high than in the wild type, suggesting the possibility of competition between starch biosynthesis and sugar supply to the fungus (Table 1).

#### Starch accumulation in the root

Vriet et al. (2010) focussed on starch accumulation in leaves for most mutants described in their study and investigated starch accumulation in roots only in selected mutants such as *pgm1-4*. Furthermore, starch dynamics in roots in response to stimuli other than the diurnal light cycle have not been investigated. Therefore, normal colonization levels of the mutants as detected in our study could be due to the fact that starch metabolism in roots or upon AM colonization is not affected. To examine this possibility we determined starch accumulation in mock inoculated and *G. margarita* colonized roots of the wild type, SL5035-7, SL4841-4 and *pgm1-4* at 4 wpi using two approaches. First, cross-sections of roots from all four genotypes were stained using the PAS reaction which reveals amyloplasts. Amyloplasts were distinguishable not only in wild type, but also in SL5035-7 and SL4841-4, showing that the defects in starch breakdown in leaves do not interfere with a regular sugar supply to the root and subsequent starch accumulation. Observations on *pgm1-4* confirmed that this mutant does not contain any amyloplasts in the root (Fig. 2d, h). Taken together the absence of amyloplasts in *pgm1-4* and the normal AM colonization levels in this mutant we conclude that starch accumulation is not required for AM colonization.

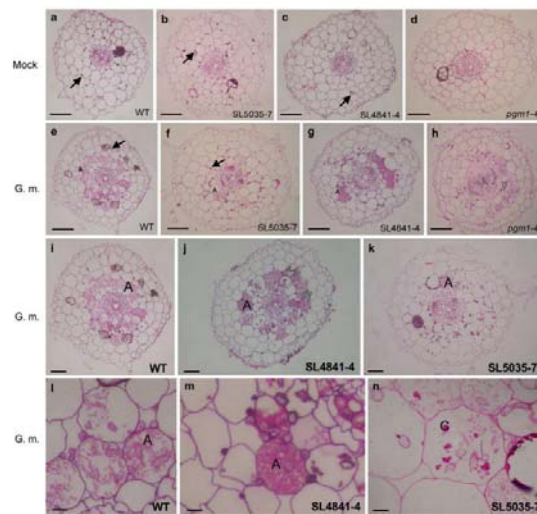


Fig. 2

a–h Cross-sections of control and mycorrhizal roots of wild type, SL5035-7, SL4841-4 and *pgm1-4* stained by the PAS reaction to visualize amyloplasts (indicated by arrows). A arbuscules. Bars 100  $\mu$ m. i–n Cross-sections of SL5035-7 and SL4841-4 roots colonized by *G. margarita*. Arbusculated cells do not contain amyloplasts (amyloplasts indicated by arrowheads). Close up photographs show that dark dots in arbusculated cells are collapsed fragments of arbuscules. A arbuscule, C collapsed fragments of arbuscules. Bars 70  $\mu$ m (i–k), 10  $\mu$ m (l, m), 6  $\mu$ m (n).



Starch granules have never been observed in arbusculated cells of wild-type plants (Bonfante 2001), but according to the previous experiment (Fig. 2a–d), mutants that are perturbed in root starch breakdown should in theory contain amyloplasts in some arbusculated cells. However, we did not find amyloplasts in arbusculated cells of the mutants SL5035-7 and SL4841-4 (Fig. 2j, k, m, n). Small dark dots in arbusculated cells that seemed at first sight to be amyloplasts were identified as collapsed fragments of fungal arbuscules (Fig. 2n, for comparison see also Bonfante-Fasolo 1984). This supports the widely accepted hypothesis that the AM fungal arbuscule and the enhanced metabolism of the arbusculated cell (Hause and Fester 2005; Franken 2010) consume all carbohydrates that enter the cell and prevent starch accumulation (Gutjahr et al. 2009). However, since our morphological analysis offers only a snapshot of the colonization events, we cannot exclude that starch might have already accumulated in a root cortex cell before it was colonized by an arbuscule.

To address this question, the number of amyloplasts in root semi-thin sections was quantified for SL5035-7 and SL4841-4 (Fig. 3a). In mock-inoculated plants, amyloplast numbers were significantly lower in SL5035-7 and SL4841-4, as compared to the wild type. In the presence of the fungus, amyloplast numbers were lower in uninfected cells of the wild type when compared to non-mycorrhizal roots, as observed previously (Gutjahr et al. 2009). As expected, in roots of SL4841-4 AM colonization led to a significant decrease of amyloplast number similar to the wild type. However, SL5035-7 showed an opposite trend, and the number of amyloplasts significantly increased upon mycorrhizal colonization.

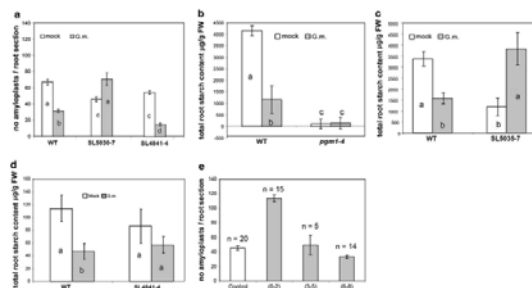


Fig. 3

Evaluation of the amyloplast numbers and whole root system starch levels upon colonization by *Gigaspora morganii* in mutants perturbed in starch metabolism. a Amyloplast numbers per root section of wild type, SL5035-7, and SL4841-4 in mock inoculated and *G. morganii* colonized roots at 4 wpi. Means and standard errors are displayed. Different letters indicate significant differences ( $P \leq 0.05$ ) as determined by the Kruskal-Wallis test,  $n = 5$ . Each plant was represented by at least five root sections. b Starch levels of whole root systems of *G. morganii* inoculated wild type and *pgm1-4* (cannot synthesize starch). c Starch levels of whole root systems of *G. morganii* inoculated wild type and SL5035-7 (cannot break down starch) mutant at 4 wpi. d Starch levels of whole root systems of *G. morganii* inoculated wild type and SL4841-4 (cannot break down starch) mutant at 4 wpi. In b, c and d, means and standard errors are shown,  $n = 5$ . For each sample two root systems were pooled. Each genotype has been separately investigated together with a set of WT plants. Different letters indicate significant differences at the 95%-level as determined by the Kruskal-Wallis test. e Distribution of amyloplasts in the mutant SL5035-7 is dependent on arbuscule numbers. Root sections were classified by number of arbuscules they contain and amyloplast numbers were recorded for each class. Means and standard errors of amyloplast numbers for each class are displayed. The number of root sections that fell into each class is displayed above the bars.

To confirm the morphological observations, we quantified starch extracted from complete root systems of all investigated genotypes. The enzymatic starch quantification assay used in our study may detect starch as well as glycogen. Therefore, as glycogen is used as sugar storage molecule by AM fungi, we first evaluated the specificity of the assay by quantifying starch in colonized and non-colonized wild type and the *pgm1-4* mutant (cannot synthesize starch). In the colonized *pgm1-4* roots, starch is absent, but the presence of the AM fungus might lead to unspecific detection of its sugar reserve molecule glycogen. However, background starch-detection levels in *pgm1-4* were very low and did not increase in presence of the AM fungus, showing that a possible contribution of fungal glycogen to the results was negligible. Furthermore, the experiment confirmed that mycorrhizal roots of wild type consistently contained less starch than non-mycorrhizal roots (Fig. 3b; Gutjahr et al. 2009).

Second, starch extracted from complete root systems of wild type, SL5035-7 and SL4841-4 at 4 wpi in the presence and in the absence of *G. margarita* was enzymatically quantified (Fig. 3c, d). In all experiments, starch levels of wild type decreased by more than half in the presence of the fungus (Fig. 3b–d). In SL5035-7, the starch level was significantly higher (Fig. 3c), while in SL4841-4 it decreased—even if not significantly—by about one third (Fig. 3d). In summary, measurement of total root-starch levels confirmed the trend shown by amyloplast numbers in the different genotypes. We conclude that SL4841-4 is able to break down starch in the root, supporting the results of Vriet et al. (2010) where turnover of starch was observed in the leaves of similar mutants. The increase in starch levels in roots of SL5035-7 upon AM colonization is consistent with an enhanced sugar transport to the root induced by AM colonization (Gutjahr et al. 2009) and an inability of SL5035-7 to break down starch in roots. These data indicate that the lesion in SL5035-7 is likely to be affecting a different process in starch turnover than that in SL4841-4.

#### Specific starch breakdown in arbusculated cells of SL5035-7

The increase of amyloplast number and starch amount in SL5035-7 upon AM colonization seems somewhat contradictory to the fact that in this mutant, amyloplasts were never observed in arbusculated cells, since starch could already accumulate in cortex cells before the formation of arbuscules. We therefore assessed whether the mutant might be able to break down starch exclusively in arbusculated cells. As an indirect measure of this ability, we quantified the number of amyloplasts in relation to the number of arbuscules in root sections of SL5035-7 by classifying the root sections according to their arbuscule content (Fig. 3e). We then quantified amyloplasts in sections belonging to the different classes. There was a clear negative relationship between arbuscule and amyloplast numbers (Fig. 3e), whereas in sections with 0–2 arbuscules, amyloplast numbers were more than twice as high as in the mock-inoculated control; in sections with 3–5 arbuscules, amyloplast numbers were similar to the mock-inoculated control and they were approximately 20% lower in sections with 6–8 arbuscules. We conclude that SL5035-7 must retain the ability to break down starch in arbusculated cells. This finding provides circumstantial evidence that carbon is not only provided directly to the AM fungus but likely also via root starch catabolism.

#### Discussion

Arbuscular mycorrhizal fungi are obligate symbionts dependent on carbon supply by their host plant for the completion of their life cycle (Smith and Read 2008). We have recently shown that changes in carbon metabolism of *Lotus japonicus* roots occur already prior to AM colonization visible as starch accumulating in response to diffusible factors released by the AM fungus (Gutjahr et al. 2009). However, it remained open whether starch accumulation is a requirement for AM colonization or simply a read-out of enhanced carbon flux into the root in response to AM fungal signals in the absence of colonization and carbohydrate consumption. By taking advantage of novel *Lotus japonicus* mutants affected in starch metabolism (Vriet et al. 2010), we demonstrate that starch can be used but is not indispensable for AM colonization. Furthermore, the different metabolic defects of the analyzed mutants lead to a diverse distribution of sugar resources in the colonized roots.

Starch accumulation is not required for arbuscular mycorrhizal colonization

The mutant *pgm1-4* is deficient in plastidial phosphoglucomutase and therefore cannot synthesize starch in any part of the plant. Quantity and morphology of AM structures do not change when starch is absent as in the roots of *pgm1-4*. The mutant directly provides the fungus with carbohydrates delivered from the shoot without a detour through starch. We have clearly demonstrated, therefore, that starch accumulation is not required for AM colonization, as already shown for the symbiosis with rhizobia (Vriet et al. 2010). However, if starch is present, it can be consumed by the AM symbiosis as will be discussed below. Elevated colonization levels of *pgm1-4* roots as compared to the wild type might even suggest competition between starch biosynthesis and carbon supply to the fungus.

Plants fix carbon and synthesize sugars only during the day when excess carbon is stored in the form of starch that could, theoretically, be used by the AM fungus during the night. Our results with *pgm1-4* provokes the hypothesis that carbon uptake by AM fungi might be coupled to the plants photosynthetic activity and take place only during daytime (at least in this mutant). The *pgm1-4* mutant provides an excellent tool to test this hypothesis in the future.

Are the mutants SL5035-7 and SL4841-4 perturbed in root starch breakdown?

Our results further show that inoculation with AM fungi can be a useful bioassay to study starch dynamics in roots of mutants defective in starch breakdown. Although the two mutants SL5035-7 and SL4841-4 displayed an equivalent starch breakdown phenotype in leaves, starch dynamics in roots could be discriminated by the arbuscular mycorrhiza assay. These mutants are known to map to different positions in the *Lotus* genome (Vriet et al. 2010) and may well encode genes representing entirely different activities affecting starch turnover.

Starch dynamics in SL5035-7 confirmed that the presence of the fungus enhances the carbon sink strength of the root sequestering sugars from the shoot (Franken 2010) that are then stored as starch, but cannot efficiently be metabolized due to the mutation in SL5035-7. The increased number of amyloplasts and starch amount (Fig. 3a, b) suggest that AM colonization induces sugar transport to the root that exceeds the immediate plant and fungal need, otherwise starch levels in SL5035-7 roots should remain constant upon mycorrhizal colonization. Alternatively, starch accumulates after an initial increase in carbon transport to the root in response to AM fungal pre-colonization signals (Gutjahr et al. 2009). On the other hand, amyloplast numbers were negatively correlated with arbuscule numbers in root sections of the same mutant. Several plant genes are known to be specifically expressed in arbusculated cells (Liu et al. 2003; Frenzel et al. 2005; Balestrini et al. 2007; Gutjahr et al. 2008; Guether et al. 2009b) and more are expected to be confirmed (Gomez et al. 2009). It is possible that a homologue of the protein mutated in SL5035-7 exists that is specifically expressed in arbusculated cells where it contributes to starch breakdown. In an alternative scenario, starch breakdown might be normal in roots of the mutant SL5035-7 but starch accumulation might be accelerated. This might be less visible in a situation where sugars are deviated away from starch biosynthesis to the fungus. The decrease in amyloplast numbers and starch levels in roots of SL4841-4 upon AM colonization clearly shows that this mutant can break down starch in roots. We also found a strong and significant decrease in starch levels and amyloplast number in the mutant *gwd1-1* defective in GLUCAN WATER DIKINASE 1 in response to AM colonization (data not shown). This suggests that AM symbioses might utilize a starch breakdown route, which is independent of GLUCAN WATER DIKINASE 1, for example via  $\alpha$ -AMYLASE (Zeeman et al. 2010). Such a hypothesis is fostered by the observed up-regulation of an  $\alpha$ -AMYLASE gene in mycorrhizal *Medicago truncatula* roots (Gomez et al. 2009).

In conclusion, our results demonstrate that starch metabolism does not influence AM colonization, and that the fungus is mostly fed by soluble sugars stemming from sucrose transported to the root by the host. This raises new questions about the circadian regulation of this nutrient supply. The negative correlation between arbuscule and amyloplast numbers in root sections of SL5035-7 suggest that starch serves as a buffer between photosynthesis and nourishment of the AM fungus. However, to address further the role of sugar metabolism and starch breakdown during the mycorrhizal symbiosis, a mutant that cannot degrade starch at all is required. This would also allow us to examine the possibility whether AM fungi possess AMYLASE activity themselves.

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